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# Inducible nitric oxide synthase-derived nitric oxide reduces vagal satiety signalling in obese mice

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**Key points**

- Obesity is associated with disrupted satiety regulation. Mice with diet-induced obesity have reduced vagal afferent neuronal excitability and a decreased afferent response to satiety signals. A low grade inflammation occurs in obesity with increased expression of inducible nitric oxide synthase (iNOS).
- Inhibition of iNOS in diet-induced obese mice restored vagal afferent neuronal excitability, increased the afferent response to satiety mediators and distention of the gut, and reduced short-term energy intake.
- A prolonged inhibition of iNOS reduced energy intake and body weight gain during the first week, and reduced amounts of epididymal fat after 3 weeks.
- We identified a novel pathway underlying disrupted satiety regulation in obesity. Blocking of this pathway might be clinically useful for the management of obesity.

**Abstract** Vagal afferents regulate feeding by transmitting satiety signals to the brain. Mice with diet-induced obesity have reduced vagal afferent sensitivity to satiety signals. We investigated whether inducible nitric oxide synthase (iNOS)-derived NO contributed to this reduction. C57BL/6J mice were fed a high- or low-fat diet for 6–8 weeks. Nodose ganglia and jejunum were analysed by immunoblotting for iNOS expression; NO production was measured using a fluorometric assay. Nodose neuron excitability and intestinal afferent sensitivity were evaluated by whole-cell patch clamp and *in vitro* afferent recording, respectively. Expression of iNOS and production of NO were increased in nodose ganglia and the small intestine in obese mice. Inhibition of iNOS in obese mice by pre-treatment with an iNOS inhibitor increased nodose neuron excitability via 2-pore-domain $K^+$ channel leak currents, restored afferent sensitivity to satiety signals and reduced short-term energy intake. Obese mice given the iNOS inhibitor daily for 3 weeks had reduced energy intake and decreased body weight gain during the first week,

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compared to mice given saline, and lower amounts of epididymal fat at the end of 3 weeks. Inhibition of iNOS or blocking the action of iNOS-derived NO on vagal afferent pathways might comprise therapeutic strategies for hyperphagia and obesity.

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## Introduction

Obesity and its related morbidity are currently one of the most prevalent public health problems. The prevalence of obesity among US adults was 36.5% during 2011–2014 (Ogden *et al.* 2015). Globally, the prevalence of being overweight and being obese combined was 36.9% for men and 38.0% for women in 2013 (Ng *et al.* 2014). Obesity is associated with health risks, such as diabetes, dyslipidaemia, hypertension, cardiovascular disease and cancer, and thus is a primary driver for increasing health costs (Sansbury & Hill, 2014; Shettar *et al.* 2017). After a protracted debate, many health organizations recently recognized obesity as a pathological state requiring clinical intervention (Bray *et al.* 2017). Bariatric surgery is effective in weight loss; however, the availability, invasiveness and frequent complications of this procedure prevent its wide application (Kim *et al.* 2011). Although there is growing evidence that neuromodulation or pharmacotherapy targeting the vagus nerve may be promising treatments (de Lartigue, 2016), our current understanding of the molecular mechanisms underlying the development and maintenance of obesity is still limited.

The vagus nerve innervating the gut plays an important role in regulating feeding behaviour and metabolism by communicating between the gut and brain. A previous study in obese mice by Daly and Park (2011) demonstrated that nodose neurons were less excitable and also that jejunum afferent sensitivity to satiety signals was attenuated. Although our recent work suggested that increased two-pore domain $K^+$ ($K_{2P}$) conductance underlies this reduced sensitivity (Park *et al.* 2018), the causal mechanisms have not been fully clarified.

Some studies suggest that NO may cause a prolonged decrease in nodose neuronal excitability (Bielefeldt *et al.* 1999) and additionally play an inhibitory neuromodulatory role on a select population of vagal afferents (Page *et al.* 2009). Both constitutive nitric oxide synthase (NOS) isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), are present in the nodose ganglia, whereas inducible nitric oxide synthase (iNOS) is not detectable under normal physiological conditions (Yamamoto *et al.* 2003). Within the gastrointestinal tract, nNOS and eNOS are found in enteric neurons and epithelial cells (Page *et al.* 2009; Sansbury & Hill, 2014) and iNOS is expressed in the epithelial cells of inflamed tissue (Kolios *et al.* 1998). iNOS has the highest capacity to generate NO in response to inflammatory stimuli.

Obesity is associated with chronic low-grade inflammation. Adipocyte hypertrophy occurs in obesity and results in the increased production of many pro-inflammatory adipokines and cytokines, such as leptin, resistin, adiponectin, tumor necrosis factor-$\alpha$, interleukin (IL)-6 and IL-1 (Hosogai *et al.* 2007; Engin, 2017). Increased leptin, hypoxia and adipocyte cell death are possible contributors to macrophage recruitment in adipose tissue (Surmi & Hasty, 2008). Recruited macrophages during a high-fat diet display inflammatory properties such as overexpression of iNOS and IL-6 (Lumeng *et al.* 2007). Interestingly, iNOS knockout mice fed a high-fat diet are protected against the development of insulin resistance (Noronha *et al.* 2005), suggesting a role for iNOS in the regulation of metabolism.

Thus, the present study aimed to test the hypothesis that iNOS-derived NO is involved in the disruption of vagal afferent signalling in diet-induced obesity. Furthermore, we hypothesized that iNOS acts via $K_{2P}$ channels because these channels have recently been implicated in the mechanism of reduced sensitivity of vagal afferents after high-fat feeding.

## Methods

### Animals and ethical approval

All experiments were performed under an approved protocol from the Queen's University Animal Care Committee (reference number 2014-1496) in accordance with guidelines from the Canadian Council for Animal Care. The authors understand the ethical principles under which *the Journal of Physiology* operates and declare that this work complies with those policies and regulations.

Diet-induced obese and control mice (male C57BL/6J; Stock Numbers: 380050 and 380056; Jackson Laboratory, Bar Harbor, ME, USA) were fed on a high-fat diet (60 kcal% fat; D12492; Research Diets, Inc., New Brunswick, NJ, USA) or a low-fat diet (10 kcal% fat; D12450B) for 6–8 weeks (starting from 6 weeks of age). High-fat fed (HFF) and low-fat fed (LFF) mice were individually housed under identical conditions with free access to water and food under a standard light/dark cycle (lights on 07.00 h). They were killed by isoflurane inhalation followed by cervical dislocation.

### Isolation of single nodose ganglion neurons

The nodose ganglia were isolated as described previously (Daly and Park *et al.* 2011).

### Western blotting

The nodose ganglia and full-sickness jejunum used for western blotting were conserved in RNALater (AM7020; Invitrogen, Valancia, CA, USA) at −20°C. Samples were lysed in radioimmunoprecipitation assay buffer containing Pierce Protease Inhibitor (catalogue no. 88666; Thermo Scientific, Waltham, MA, USA) cocktail and homogenized using three 10 s bursts of sonication at 1 min intervals. Following a 30 min incubation on ice and a 20 min spin at 16,000 *g*, supernatants were collected and the protein concentration was determined using a BCA Protein Assay kit (catalogue no. 23225; Thermo Scientific). Next, 50 $\mu$g of proteins were separated by SDS-PAGE (6–15% gradient) at 150 V. Samples were transferred to Immobilon polyvinylidene difluoride membrane (IPFL00010; Millipore, Billerica, MA, USA) at 25 V in transfer buffer (25 mM Tris, 190 mM glycine and 10% methanol) overnight at 4°C. Membrane was blocked with 5% non-fat milk for 2 h and incubated with primary antibodies (rabbit polyclonal iNOS antibody, dilution 1:500; NB300605; Novus Biologicals, Littleton, CO, USA; mouse monoclonal $\beta$-actin antibody, dilution 1:5000; A5316; Sigma, St Louis, MO, USA) overnight at 4°C, followed by three washes with Tris-buffered saline with Tween 20 (TBST). HRP-conjugated secondary antibody (goat anti-rabbit IgG, dilution 1:5000; catalogue no. 7074; Cell Signaling Technology, Beverly, MA, USA; goat anti-mouse IgG, dilution 1:5000; catalogue no. 31430; Invitrogen) was added to the membrane, followed by a 1 h incubation at room temperature and three washes with TBST. Immunoreactive bands were visualized by chemiluminescence using SuperSignal West Pico ECL reagents (catalogue no. 34087; Thermo Scientific) or Luminata Forte Western HRP substrate (WBLUF0100; Millipore). Images were acquired and analysed using a ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA) and Image Lab software, version 5.1 (Bio-Rad). Band boundaries of LFF and HFF samples were adjusted to be identical.

### Nitrate/nitrite fluorometric assay

The nitrate and nitrite concentration in the jejunum, serum and culture media of nodose ganglion neuron from both LFF and HFF mice was measured using a fluorometric assay kit (780051; Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the manufacturer's instructions. Serum and jejunum were collected from mice with/without L-$N^6$-(1-iminoethyl) lysine dihydrochloride (L-NIL; I.P. injection, 10 mg kg$^{-1}$) treatment. Nodose neurons were incubated in media with/without $N^{\omega}$-propyl-L-arginine ($N^{\omega}$, 100 nM) or L-NIL (10 $\mu$M) for 30 min before media collection. About 30 mg of jejunum tissue was collected in PBS, homogenized using a motor-driven pestle (FS749540-0000; Fisherbrand, Fisher Scientific Co., Pittsburgh, PA, USA) and ultrafiltered using a 10 kDa ultrafilter (UFC501024; Millipore). The same amount of sample (1 ml) as that used for the serum and nodose ganglion culture media was collected for assay. Total nitrate and nitrite concentration was determined using the nitrate standard curve (ranging from 0.06 to 3.85 $\mu$M). For jejunum, the concentration of nitrate/nitrite was normalized to the weight of tissue collected.

### Multi-unit afferent nerve recordings

The nerve activity of afferents innervating mouse small intestine was recorded as described previously (Daly and Park *et al.* 2011). Most drugs were perfused into the bath, for either a brief 1 min exposure or a 15 min continuous perfusion, whereas only glucagon-like peptide 1 (GLP-1) agonist exendin-4 (ex-4) was delivered intraluminally for 10 min. Based on work from our group and another group, the GLP-1 response has a long latency (Kakei *et al.* 2002) and minimal effect when given by bath perfusion; thus, we applied ex-4 intraluminally for 10 min to reduce the amount of drug needed (given the much lower intraluminal perfusion rate) and to be able to observe a reliable response that could be compared between LFF and HFF groups.

Single unit analysis was performed using the spike sorting function of Spike2 (Cambridge Electronic Design Ltd, Cambridge, UK) to discriminate the activities of individual afferent units. Based on their sensitivity to ramp distention, afferent units were classified into three subpopulations: low threshold (LT), wide dynamic range (WDR) and high threshold (HT) (Rong *et al.* 2004). LT afferents responded to low pressure and plateaued after the first few increases in mmHg. WDR afferents also had a low threshold, although their firing rate continued to increase with pressure. HT afferents were defined as units with a threshold over 15 mmHg. Drug responses were assessed by mean change in afferent discharge over a 30 s period at the peak compared to a 30 s baseline period. Drug responses were normalized against the number of responsive units in that nerves. Distention responses were analysed using a customer-made script and expressed as the increase in firing frequency with increased pressure. To compare distention responses among different preparations, firing frequency was normalized against the number of units. To compare distention responses within the same preparations, firing frequency was normalized against the peak firing rate of the control distention.

### Whole-cell patch clamp recordings

Whole-cell patch clamp recordings were performed as described previously (Daly and Park *et al.* 2011). Nodose ganglion neurons were randomly selected for recording, based on our previous work demonstrating that obesity decreases the excitability of both randomly selected neurons and small intestine-projecting neurons (Daly and Park *et al.* 2011). Neurons were recorded for 10–15 min and recordings were terminated if membrane potential/access resistance changed during this time. $K_{2P}$ currents were isolated by ion replacement and a pharmacological method as described previously (Park *et al.* 2018). The $K_{2P}$ channels do not have time-dependent kinetics (Lesage *et al.* 1996) and thus a ramp protocol (−90 to 30 mV, 400 ms, 10 mV increment, $V_h = -60$ mV, 2.5 kHz sampling) was applied. Junction potentials (10.1 mV) were corrected after recording. Recordings were analysed using Clampfit, version 10.0 (Axon Instruments Inc., Foster City, CA, USA) and Origin (OriginLab, Northampton, MA, USA).

### Food intake measurement

Food intake was monitored using TSE LabMaster Home Cage System (TSE Systems, Inc., Chesterfield, MO, USA). Mice were allowed to acclimate to the metabolic cages for at least 3 days prior to any treatment. Energy intake and meal pattern were analysed using the same software for monitoring. A meal should be separated from other feeding events by more than 10 min and the minimal meal size was defined as 0.03 g. Energy intake was calculated as food intake multiplied by energy density (5.24 and 3.85 kcal $g^{-1}$ for the high- and low-fat diet, respectively).

To evaluate the influence of iNOS on short-term energy intake, LFF and HFF mice received an I.P. injection of saline (equal volume as L-NIL) at 18.00 h followed by an injection of L-NIL (10 mg $kg^{-1}$), 24 h later. An I.P. injection was performed using a 1 mL syringe and a 26-G needle. Feeding patterns during 24 h after treatment were compared with saline injection. To evaluate the long-term effects of iNOS suppression on energy intake and adiposity, HFF mice were divided into two groups, receiving one daily I.P. injection of L-NIL or an equivalent volume of saline for 3 weeks, respectively. Injection sites were alternated daily between the left and right lower abdominal quadrants. Epididymal fat pads were weighed after death of the mice.

### Drugs

All drugs were prepared as stock solution, kept frozen at −20°C and diluted to their final concentration in either Krebs buffers in afferent recording or Hepes buffer in patch clamp immediately prior to application. Regrading commercial sources: L-NIL (catalogue no. 80310), 5-hydroxytryptamine (5-HT; catalogue no. 14332), exendin-4 (acetate; catalogue no. 11096), $N^{\omega}$ (catalogue no. 80587), *N*-([3-(aminomethyl) phenyl]methyl)ethanimidamide dihydrochloride (1400W; catalogue no. 81520), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; catalogue no. 81410) and sildenafil citrate (14008) were purchased from Cayman Chemical (Cayman Chemical Company, Ann Arbor, MI, USA); sodium nitroprusside dihydrate (SNP; catalogue no. 71778) was obtained from Sigma; and cholecystokinin octapeptide sulphated (CCK-8S; catalogue no. ab120209) was obtained from Abcam (Abcam, Cambridge, MA, USA).

### Statistical analysis

All data were expressed as the mean ± SD unless otherwise stated. A significant difference was determined using Student's *t* test (two-tailed), or a one- or two-way ANOVA with a Bonferroni test as appropriate. *N* refers to the number of animals, and *n* indicates the number of cells or samples. $P < 0.05$ was considered statistically significant. Significance was defined as: $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). An absence of asterisks indicates no significance or cases where it is not applicable.

## Results

### Increased iNOS expression and NO production in HFF mice

Immunoblotting with an anti-iNOS antibody detected protein bands of ∼130 kDa in all nodose ganglion and jejunum samples from HFF mice (Fig. 1*A*). Bands were barely visible in all three nodose ganglion pools and two out of eight jejunum samples from LFF mice. Because iNOS protein abundance in the nodose ganglion was much lower than the jejunum, nodose ganglia from four mice were pooled and visualized using a more sensitive HRP substrate. iNOS expression (normalized against $\beta$-actin) was increased in both the nodose ganglia (31.7 ± 5.7% *vs.* 15.8 ± 3.2%, $P < 0.05$, unpaired *t* test, $n = 3$ pools, $N = 12$) (Fig. 1*B*) and jejunum (85.4 ± 20.5% *vs.* 48.2 ± 25.9%, $P < 0.01$, unpaired *t* test, $N = 8$) (Fig. 1*C*) from HFF mice.

Total nitrate/nitrite was measured as an index for NO production using a fluorometric assay kit. There was a statistically significant interaction between the effects of diet and L-NIL treatment (a selective iNOS inhibitor, 10 mg $kg^{-1}$, I.P. injection) on nitrate/nitrite level in both jejunum [$F_{1,26} = 5.282$, $P < 0.05$, B coefficient = −0.19, 95% confidence interval (CI): −0.36 to −0.02, two-way ANOVA with Bonferroni test, $N \geq 8$] (Fig. 1*D*) and serum ($F_{1,20} = 22.89$, $P < 0.001$, B coefficient = −2.66, 95% CI: −3.82 to −1.50, $N = 6$) (Fig. 1*E*). Simple main effects analysis with Bonferroni *post hoc* tests revealed that untreated HFF mice had a higher nitrate/nitrite level

in the jejunum homogenate (0.35 ± 0.17 *vs.* 0.17 ± 0.10 $\mu$M mg$^{-1}$, $P < 0.05$) and serum (3.23 ± 1.09 *vs.* 0.29 ± 0.08 $\mu$M, $P < 0.001$) compared to untreated LFF mice; in addition, L-NIL had no significant effect in LFF mice ($P > 0.999$) but did decrease NO production significantly in HFF mice in both jejunum (0.35 ± 0.17 *vs.* 0.14 ± 0.06 $\mu$M mg$^{-1}$, $P < 0.01$) and serum (3.23 ± 1.09 *vs.* 0.88 ± 0.68 $\mu$M, $P < 0.001$). Similarly, there was a statistically significant interaction between the effects of diet and L-NIL or $N^{\omega}$ (100 nM, nNOS inhibitor) treatment on NO

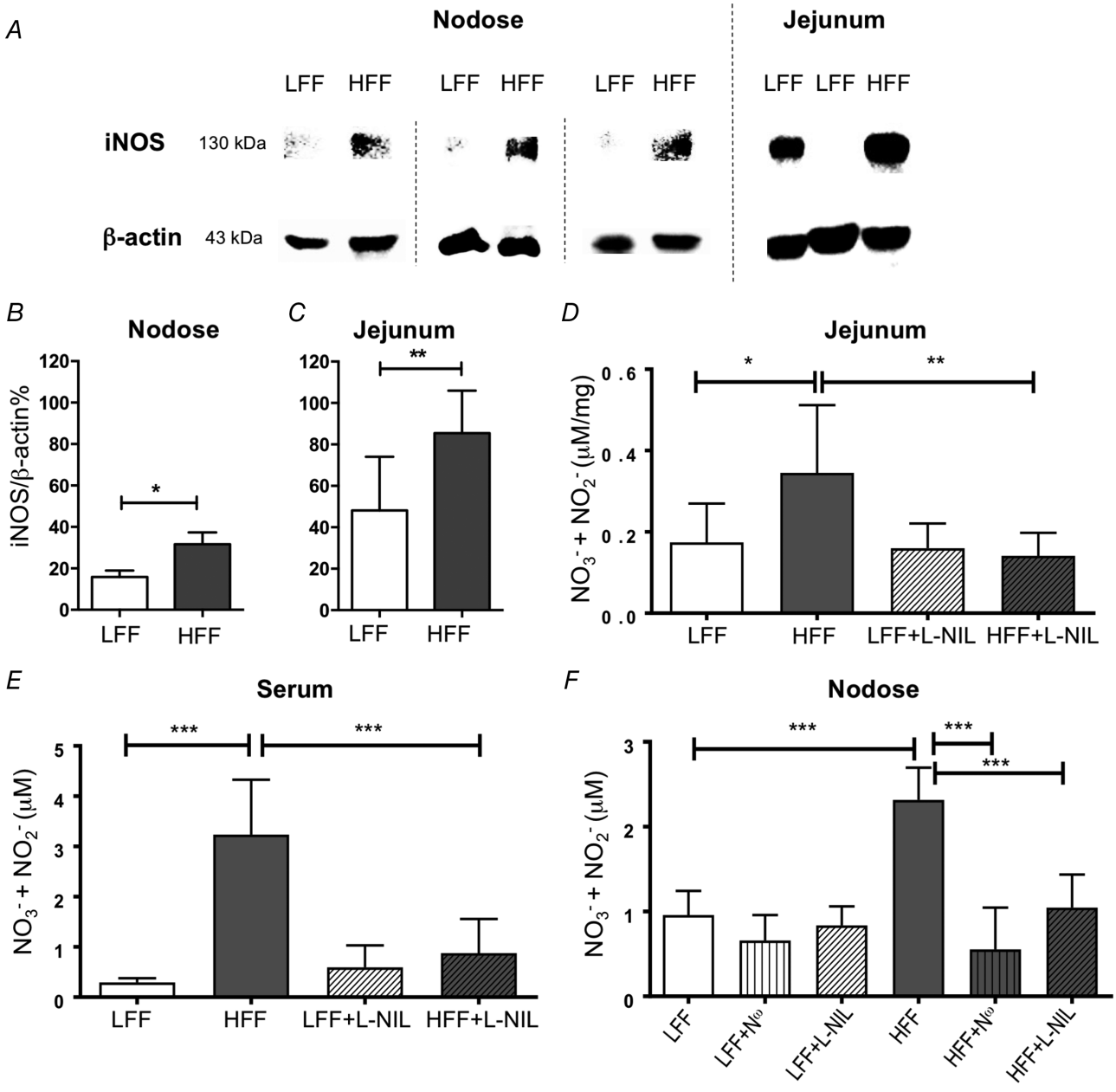


**Figure 1. iNOS expression and NO production were increased in HFF mice**
*A*, total iNOS protein in nodose ganglion and jejunum visualized on SDS-PAGE gel by western blotting. *B* and *C*, iNOS expression was increased in HFF mice nodose ganglia (*B*) ($P < 0.05$, $n = 3$ pools, each containing nodose ganglia from four mice, unpaired *t* test) and jejunum (*C*) ($P < 0.01$, $N = 8$). *D* and *E*, total nitrate/nitrite was increased in the jejunum (*D*) ($P < 0.05$, $N \geq 8$, two-way ANOVA with Bonferroni test) and serum (*E*) ($P < 0.001$, $N = 6$) from HFF mice. L-NIL treatment (10 mg/kg, I.P. injection) reversed this change in both jejunum (*D*) ($P < 0.01$, $N \geq 7$) and serum (*E*) ($P < 0.001$, $N = 6$). *F*, total nitrate/nitrite was significantly increased in culture media of nodose neurons from HFF mice compared to LFF mice ($P < 0.001$, $N = 6$, two-way ANOVA with Bonferroni test). This augmentation in HFF nodose neurons was significantly attenuated by $N^{\omega}$ (100 nM, $P < 0.001$, $N = 6$) and L-NIL (10 $\mu$M, $P < 0.001$, $N = 6$).

production in the culture media incubated with nodose neurons [$F_{2,30} = 14.037$, $P < 0.001$, B coefficient (L-NIL) = −1.146, 95% CI: −1.739 to −0.553, B coefficient ($N^{\omega}$) = −1.463, 95% CI: −2.056 to −0.969, $N = 6$] (Fig. 1*F*). Simple main effects analysis revealed that HFF mice had a higher nitrate/nitrite level in the culture media incubated with nodose neurons (2.32 ± 0.38 *vs.* 0.96 ± 0.28 μM, $P < 0.001$) and this was reduced by both L-NIL ($P < 0.001$) and $N^{\omega}$ ($P < 0.001$).

### Inhibition of NO signalling increased membrane excitability in nodose neurons from HFF mice

High-fat diet feeding resulted in decreased excitability of nodose ganglion neurons (Daly and Park *et al.* 2011). Inhibition of NO synthase for 30 min increased the excitability in HFF nodose neurons. $N^{\omega}$ (100 nM), L-NIL (10 μM or I.P. 10 mg kg$^{-1}$) and 1400W (10 nM, a selective iNOS inhibitor) significantly decreased the rheobase in HFF but not LFF neurons (HFF, 112.1 ± 54.7 pA, $n = 14$ *vs.* HFF+$N^{\omega}$, 68.0 ± 13.2, $n = 10$, $P < 0.01$; HFF+L-NIL, 75.5 ± 18.6, $n = 11$, $P < 0.05$; HFF+L-NIL (I.P.), 70.0 ± 17.5, $n = 14$, $P < 0.01$; HFF+1400W, 53.3 ± 17.3, $n = 9$, $F_{6,73} = 5.894$, $P < 0.001$, one-way ANOVA with Bonferroni test) (Fig. 2*D* and *E*). The number of action potentials at 2 × rheobase was significantly increased by the iNOS inhibitor, 1400W in HFF but not LFF neurons (HFF, 1.4 ± 0.5, $n = 14$ *vs.* HFF+1400W, 2.9 ± 2.3, $n = 9$, $F_{6,73} = 2.472$, $P < 0.05$, one-way ANOVA with Bonferroni test) (Fig. 2*D* and *E*). Input resistance was significantly increased by NO synthase inhibitors in HFF neurons (HFF, 285.3 ± 122.6 MΩ, $n = 14$ *vs.* HFF+$N^{\omega}$, 657.6 ± 394.5, $n = 10$, $P < 0.001$; HFF+L-NIL (I.P.), 480.4 ± 202.0, $n = 14$, $P < 0.05$; HFF+1400W, 515.7 ± 90.5, $n = 9$, $F_{6,73} = 5.429$, $P < 0.05$, one-way ANOVA with Bonferroni test) (Fig. 2*D* and *E*). One of the targets for NO is soluble guanylate cyclase (sGC), the activation of which results in the accumulation of guanosine 3′,5′- cGMP. Inhibitions of sGC by ODQ increased the excitability of HFF neurons. ODQ (10 μM, 30 min incubation) significantly decreased the rheobase in HFF neurons (HFF, 112.1 ± 54.7 pA, $n = 14$ *vs.* HFF + ODQ, 54.0 ± 20.7 pA, $n = 10$, $P < 0.001$) (Fig. 2*E*) and tended to increase input resistance (HFF, 285.3 ± 122.6 MΩ, $n = 14$ *vs.* HFF+ODQ, 463.7 ± 90.5 MΩ, $n = 10$, $P = 0.153$) and the number of action potentials at 2 × rheobase (HFF, 1.4 ± 0.5, $n = 14$ *vs.* HFF+ODQ, 2.4 ± 1.0, $n = 10$, $P = 0.193$). Sildenafil (1 μM), an inhibitor of the enzyme that promotes degradation of cGMP, did not significantly affect nodose neuron excitability.

### iNOS inhibition decreased $K_{2P}$ conductance in nodose neurons from HFF mice

Figure 3*A* shows the representative *I–V* relationships in the presence of blockers of other ion channels (10 mM TEA, 0.5 mM 4-AP, 200 μM $Ba^{2+}$, 200 μM $Cd^{2+}$ and 25 μM ZD7288), showing the remaining leak $K^+$ ($K_{2P}$) current on nodose neurons from HFF mice with or without L-NIL treatment. Leak conductance was determined by a linear fit to the ohmic region (−100 to −50 mV) of the *I–V* relationship. The current traces in this membrane potential range were well fitted by linear function, indicating leak conductance. There was a statistically significant interaction between the effects of diet and L-NIL treatment on mean leak conductance (gLeak) [$F_{2,72} = 3.966$, $P < 0.05$, B coefficient (L-NIL, I.P.) = −0.257, 95% CI: −0.443 to −0.072, B coefficient (L-NIL) = −0.138, 95% CI: −0.316 to −0.040, two-way ANOVA with Bonferroni test, $n \geq 10$] (Fig. 3*B*). Simple main effects analysis revealed that gLeak was significantly increased in HFF neurons (0.309 ± 0.146 nS, $n = 17$ *vs.* 0.466 ± 0.226, $n = 18$, $P < 0.05$) and was reversed by inhibition of iNOS [HFF+L-NIL (I.P.), 0.274 ± 0.095, $n = 10$, $P < 0.05$].

### iNOS inhibition potentiated afferent mechanosensitivity in HFF mouse jejunum

Mechanosensitivity of intestinal afferent nerves was examined by *in vitro* afferent nerve recording on isolated jejunum segments. Ramp distention of preparations induced a typical biphasic increase in afferent discharge (Fig. 4*A* and *B*), corresponding to the activation of different afferent subpopulations. Mechanosensitive units were classified into LT, WDR and HT subpopulations as described in the Methods. Mechanically insensitive units identified in this set-up were limited (<2%). It is generally accepted that HT units are exclusively spinal afferents sensing noxious stimuli, whereas LT afferents can be of both vagal and spinal origin. Controversy remains regarding the origin of WDR afferents. HFF mice that received an I.P. injection of L-NIL (10 mg kg$^{-1}$) showed an augmented afferent response to distention compared to non-treated HFF mice ($F_{1,24} = 4.423$, $P < 0.05$, two-way ANOVA with Bonferroni test, $N = 12$) (Fig. 4*C*). Single unit analysis revealed that this increase was contributed to by LT ($P < 0.05$) (Fig. 4*E*) and WDR ($P = 0.094$) afferents. No significant change was observed in LFF mice ($F_{1,25} = 3.303$, $P = 0.081$, $N = 13$) (Fig. 4*D*).

### iNOS inhibition potentiated satiety signalling in HFF mouse small intestine

The jejunal or ileal afferent response to satiety mediators was investigated using the same recording technique. Only Ex-4 (GLP-1 agonist) related experiments were performed on the ileum. In agreement with our previous study, HFF mice had impaired afferent sensitivity to 5-HT (10 μM), Ex-4 (100 nM) and CCK (100 nM) (Fig. 5) compared to LFF mice. There was a statistically significant interaction between the effects of diet and L-NIL treatment (10 mg kg$^{-1}$, I.P. injection) on the afferent response to

5-HT ($F_{1,30} = 6.405$, $P < 0.05$, B coefficient = 2.759, 95% CI: 0.533 to 4.986, two-way ANOVA with Bonferroni test, $N \geq 7$) (Fig. 5*A*) and Ex-4 ($F_{1,26} = 10.455$, $P < 0.01$, B coefficient = 4.423, 95% CI: 1.611 to 7.235, two-way ANOVA with Bonferroni test, $N \geq 7$) (Fig. 5*B*) but not CCK ($F_{1,28} = 3.254$, $P = 0.082$, B coefficient = 3.221, 95% CI: −0.437 to 6.879, two-way ANOVA with Bonferroni test, $N \geq 7$) (Fig. 5*C*). L-NIL significantly increased the afferent response to 5-HT in HFF mice (6.4 ± 1.9 *vs.* 3.5 ± 0.8 spikes $s^{-1}$, $P < 0.01$) (Fig. 5*A*) by potentiating LT (6.6 ± 2.8 *vs.* 4.0 ± 1.0 spikes $s^{-1}$, $P < 0.05$) and WDR units (5.0 ± 2.9 *vs.* 2.4 ± 1.5 spikes $s^{-1}$, $P = 0.063$). In line with 5-HT, Ex-4 sensitivity was enhanced by L-NIL in HFF mice (5.3 ± 1.6 *vs.* 2.2 ± 1.1 spikes.$s^{-1}$, $P < 0.05$) (Fig. 5*B*) and this was also contributed to by LT (5.6 ± 1.8 *vs.* 2.1 ± 1.3 spikes $s^{-1}$, $P < 0.05$). CCK sensitivity in HFF mice was slightly increased by L-NIL, although it was not statistically significant (8.3 ± 2.5 *vs.* 6.5 ± 1.6 spikes $s^{-1}$, $P = 0.412$)

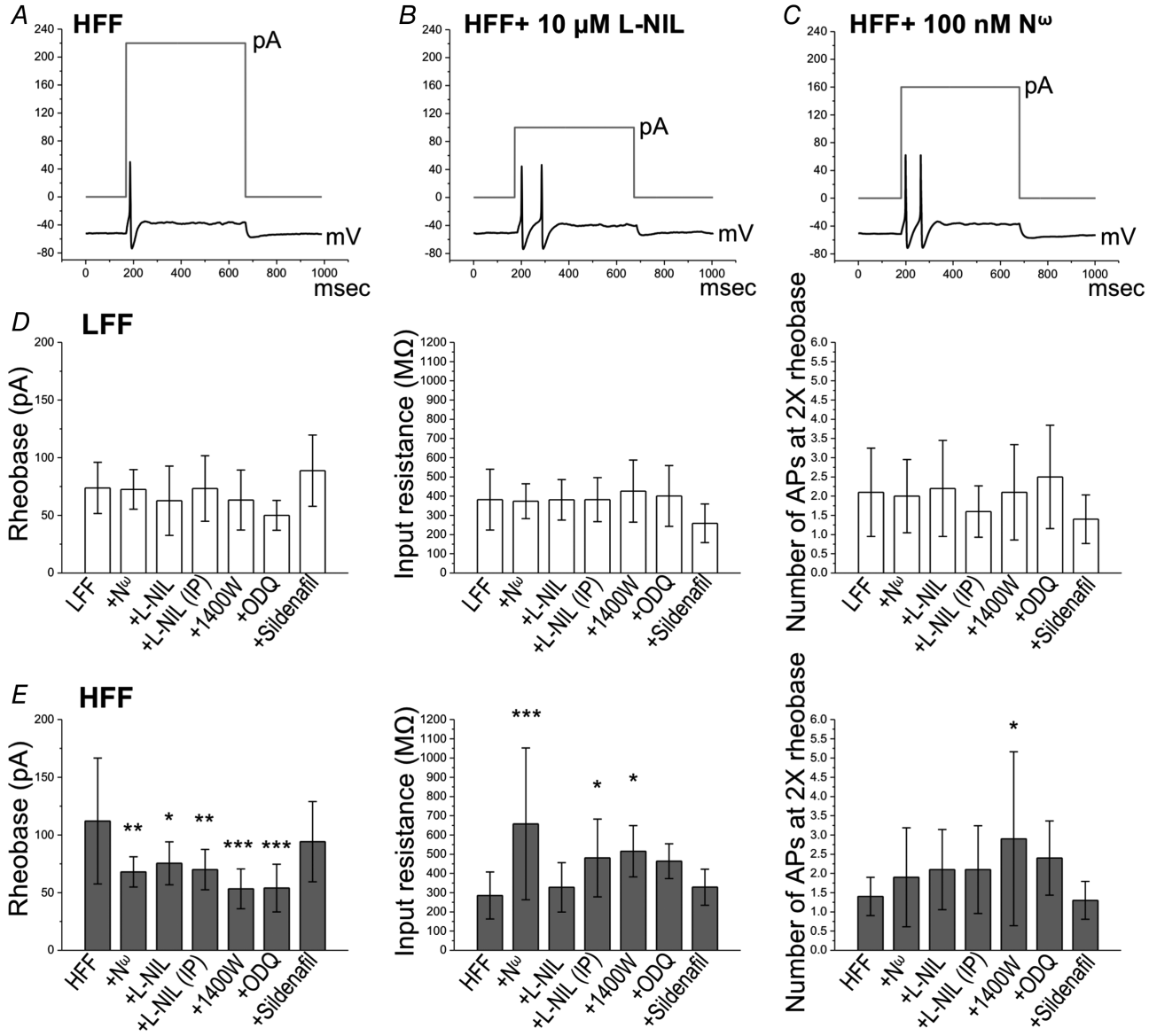


**Figure 2. Inhibition of NO signalling pathway increased excitability of nodose neurons from HFF mice**
Representative action potential firing elicited by 2 × rheobase current pulse (grey) in HFF nodose neurons (*A*), HFF+10 μM L-NIL (*B*) and HFF+100 nM $N^{\omega}$ (*C*). Histograms illustrating mean rheobase, input resistance and number of action potentials at 2 × rheobase in nodose neurons from LFF (*D*) and HFF (*E*) mice. Inhibition of NOS by $N^{\omega}$ (100 nM, $P < 0.01$), L-NIL (10 μM, $P < 0.05$), L-NIL (I.P. 10 mg $kg^{-1}$, $P < 0.01$) and 1400W (10 nM, $P < 0.001$), as well as blockade of sGC by ODQ (10 μM, $P < 0.001$), significantly decreased rheobase in HFF neurons. $N^{\omega}$ ($P < 0.001$), L-NIL (I.P., $P < 0.05$) and 1400W ($P < 0.05$) significantly increased input resistance in HFF neurons. 1400W ($P < 0.05$) significantly increased the number of action potential in HFF neurons.

(Fig. 5*C*). However, WDR afferents with L-NIL treatment showed a significant increase in CCK sensitivity (10.4 ± 3.1 *vs.* 4.1 ± 1.6 spikes $s^{-1}$, $P < 0.01$). No significant change was seen in LFF mice for all subpopulations. HT afferents were minimally involved in this satiety signalling (data not shown), in line with evidence that these satiety mediators mainly target vagal afferents (Richards *et al.* 1996; Hillsley & Grundy, 1998).

### Exogenous NO inhibited selected afferent signalling in LFF mouse jejunum

To confirm the inhibitory effects of NO on afferent sensitivity, SNP (10 $\mu$M), a NO donor, was perfused into the organ bath for 15 min. Overall, SNP significantly inhibited the afferent response to 5-HT ($F_{1,12} = 10.89$, $P < 0.01$, two-way ANOVA with Bonferroni test, $N = 7$) (Fig. 6*A*), particularly in LFF mice (5.2 ± 2.0 *vs.* 3.2 ± 2.2 spikes $s^{-1}$, $P < 0.05$) by inhibition on LT afferents (7.8 ± 3.1 *vs.* 4.6 ± 2.0 spikes $s^{-1}$, $P < 0.01$). No significant change was observed in WDR and HT afferents (data not shown). Afferent sensitivity to distention was inhibited by SNP in LFF instead of HFF mice ($F_{1,12} = 32.27$, $P < 0.001$ and $F_{1,12} = 0.436$, $P = 0.522$, two-way ANOVA with Bonferroni test, $N = 13$) (Fig. 6*C* and *D*) and only WDR units in LFF mice showed significant inhibition ($P < 0.01$) (Fig. 6*E*). Conversely, SNP did not change the CCK response in both LFF and HFF mice ($F_{1,11} = 0.609$, $P = 0.452$, two-way ANOVA with Bonferroni test, $N \geq 6$) (Fig. 6*B*). To explain the discrepancy between 5-HT and CCK data, the effects of SNP on individual unit sensitivity in LFF mice were analysed by single unit analysis (Fig. 6*F*) and a noteworthy difference was found in LT afferents. Accordingly, 88.2% of LT units (15 out of 17) in 5-HT experiments were inhibited by SNP, whereas, in CCK experiments, SNP showed diverse effects on LT afferents (43.5% excitation *vs.* 26.1% inhibition, total units 23). NO donor SIN-1 (0.5 mM) also decreased the excitability of cultured nodose neurons from LFF mice, as indicated by increased rheobase (57.9 ± 6.7 pA, $n = 14$ *vs.* 79.3 ± 6.6, $n = 15$, $P < 0.05$, unpaired *t* test).

### iNOS inhibition reduced food intake and adiposity in HFF mice

Feeding patterns during 24 h after I.P. injection of L-NIL were compared with saline. Saline injection did not change energy intake in both groups of mice (HFF: $P = 0.989$; LFF:

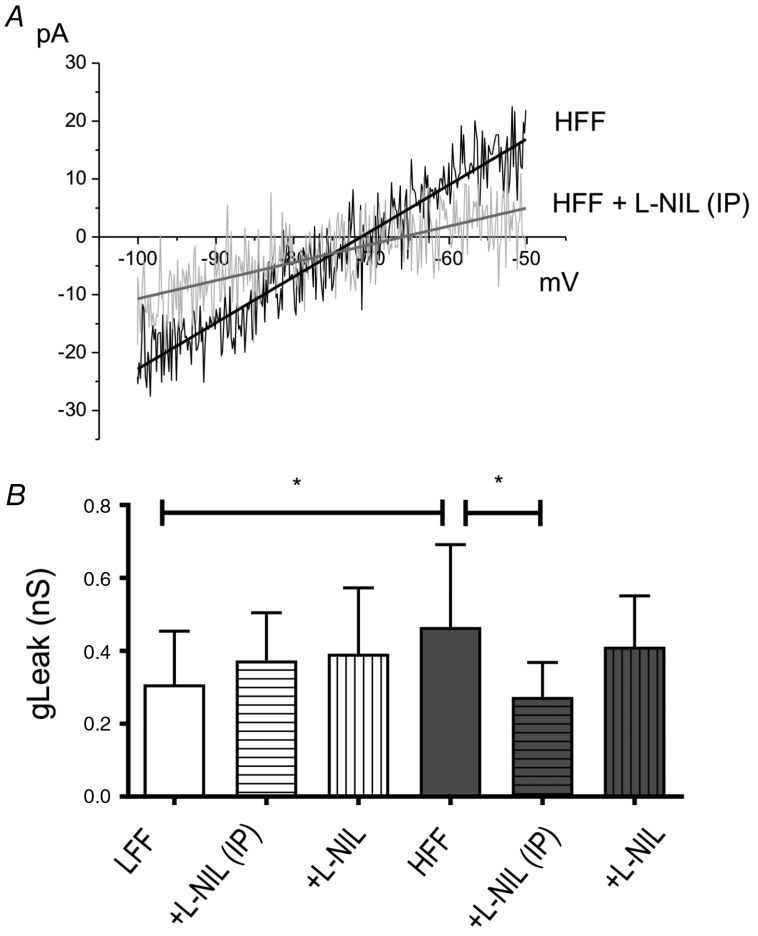


**Figure 3. Inhibition of iNOS/NO signalling pathway decreased $K_{2P}$ conductance**
*A*, representative $K_{2P}$ currents were evoked by ramp pulses in the presence of blockers in neurons from HFF (black) and L-NIL-pretreated (10 mg $kg^{-1}$, I.P. injection) HFF mice (grey). *I–V* relationships showed that L-NIL elicited a decrease in inward current of the ohmic region ($P < 0.05$). *B*, histogram showing mean leak conductance (gLeak) in control and L-NIL-pretreated (10 $\mu$M, incubation for 30 min. or 10 mg/kg, I.P.) neurons from LFF and HFF mice, respectively.

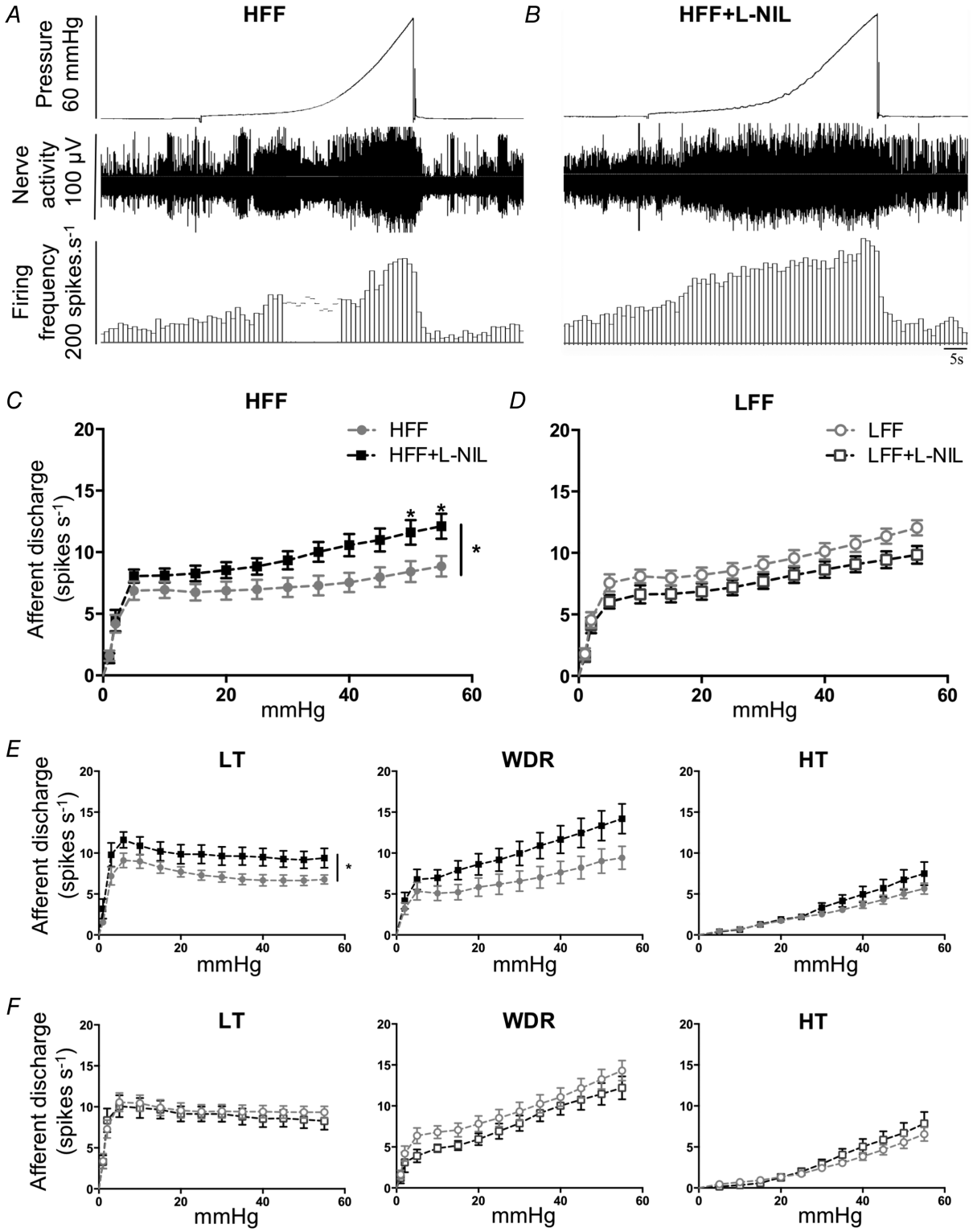


**Figure 4. iNOS inhibition potentiated the afferent response to distention in HFF mice jejunum**
*A* and *B*, representative traces showing multi-unit afferent response to distention in jejunum segments from HFF mice (*A*) and L-NIL-pretreated (10 mg $kg^{-1}$, I.P. injection) HFF mice (*B*). *C* and *E*, effects of L-NIL treatment on mechanosensitivity of total afferents and subpopulations in HFF mouse jejunum. L-NIL significantly potentiated the total afferent response to increased intraluminal pressure (*C*) ($P < 0.05$, two-way ANOVA with Bonferroni test, $N = 12$). LT ($P < 0.05$) and WDR ($P = 0.094$) instead of HT ($P = 0.284$) afferents contributed to this potentiation. *D* and *F*, no significant change was observed in LFF mice in all units (*D*) and all subpopulations (*F*) ($N = 13$).

$P = 0.451$; paired $t$ test, $N = 7$). There was a statistically significant interaction between the effects of diet and L-NIL treatment on energy intake ($F_{1,12} = 12.81$, $P < 0.01$, two-way ANOVA with Bonferroni test, $N = 7$) (Fig. 7*A*). HFF mice had a higher daily energy intake compared to LFF mice (11.57 ± 1.66 *vs.* 9.39 ± 1.28 kcal, $P < 0.05$), indicating the presence of hyperphagia. Energy intake in HFF mice was significantly reduced after L-NIL injection (11.57 ± 1.66 *vs.* 9.51 ± 1.75 kcal, $P < 0.05$), whereas LFF was not significantly changed, despite a tendency to increase ($P = 0.168$). No difference was observed in average meal size between two groups of mice and between saline and L-NIL treatment (Fig. 7*B*). L-NIL increased inter-meal interval in HFF mice (67 ± 7 *vs.* 85 ± 14 min., $P < 0.05$, two-way ANOVA with Bonferroni test) (Fig. 7*C*). HFF mice showed higher energy intake than LFF mice in the dark phase (9.33 ± 1.38 *vs.* 7.18 ± 1.34 kcal, $P < 0.05$), although without any significant difference in the light phase (2.24 ± 1.29 *vs.* 2.21 ± 0.62 kcal, $P = 0.96$). L-NIL treatment appeared to reduce the energy intake of

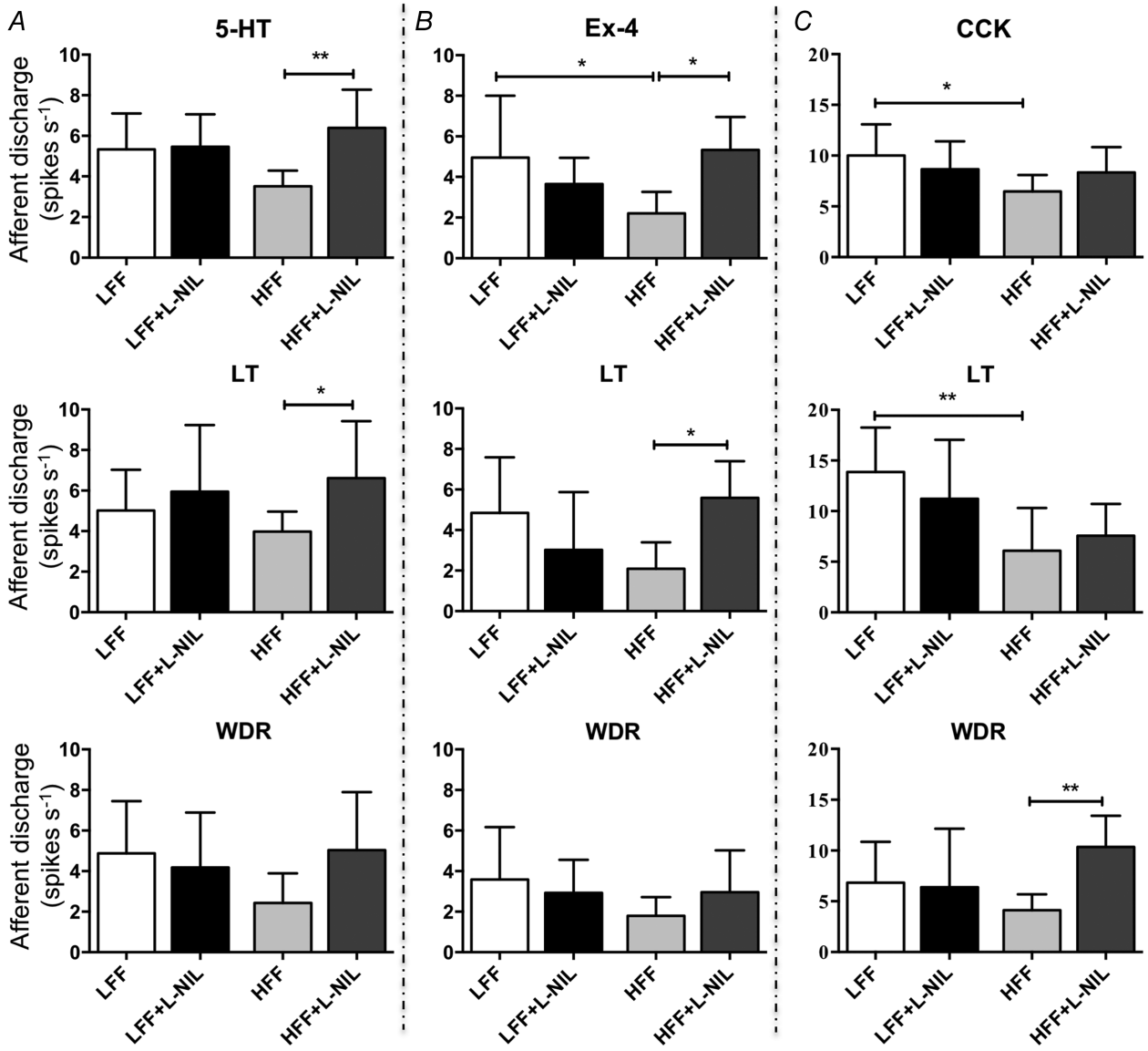


**Figure 5. iNOS inhibition potentiated the afferent response to satiety mediators in HFF mice**
*A*, effects of L-NIL on 5-HT sensitivity of jejunum afferents and subpopulations. L-NIL treatment (10 mg kg$^{-1}$, I.P. injection) resulted in a significant increase in total afferent response to 10 $\mu$M 5-HT ($P < 0.01$, two-way ANOVA with Bonferroni test, $N = 9$), as well as in LT ($P < 0.05$) and WDR ($P = 0.063$) afferents, in HFF mice. *B*, effects of L-NIL on GLP-1 signalling in ileum afferents and subpopulations. I.P. injection of L-NIL in HFF mice augmented the afferent response to 100 nM Ex-4 in all units ($P < 0.05$, $N = 8$) and LT afferents ($P < 0.05$). *C*, afferent sensitivity to CCK (100 nM) was not significantly changed by L-NIL in HFF mice jejunum ($P = 0.412$, $N = 8$), whereas only WDR afferents were significantly potentiated ($P < 0.01$).

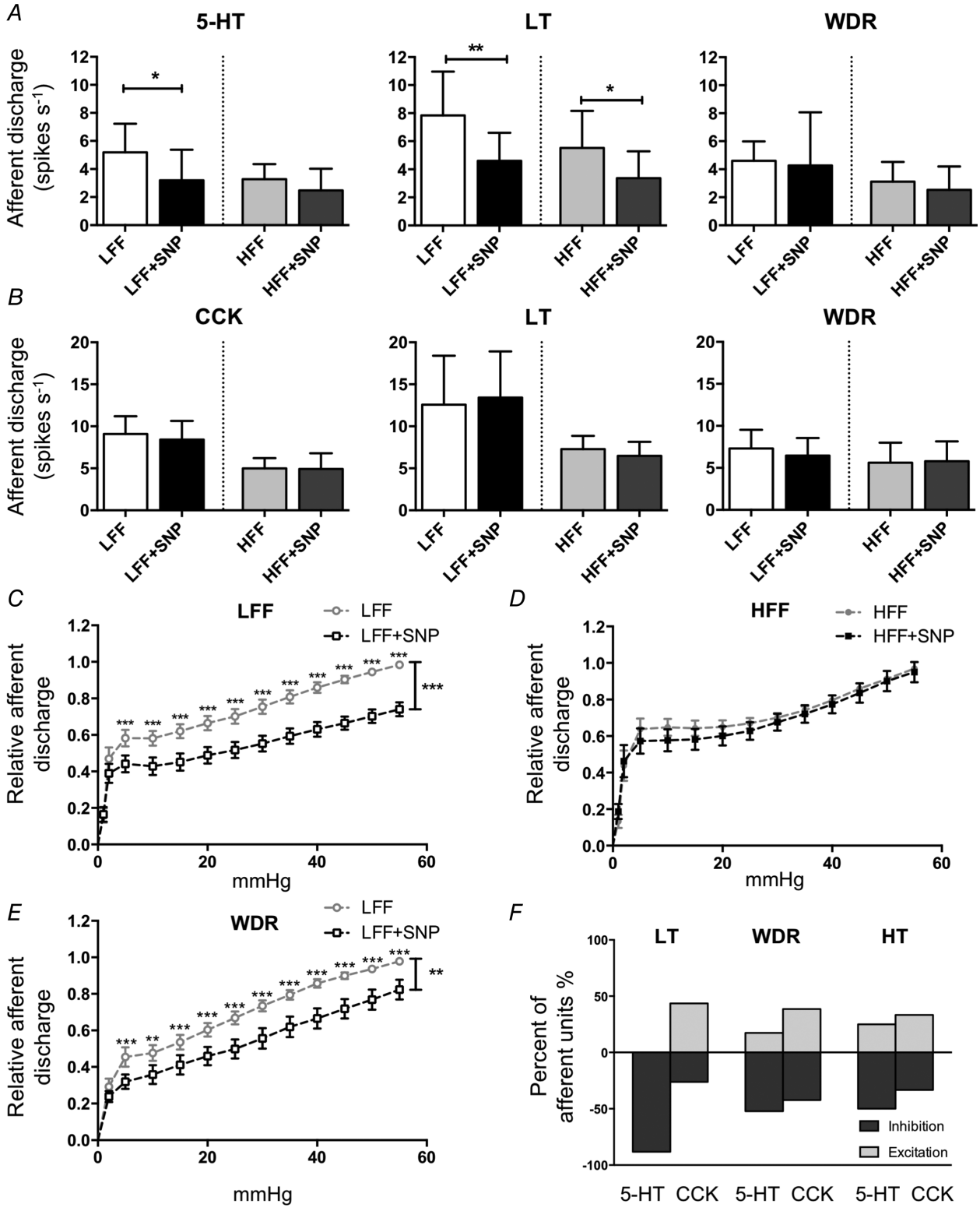


**Figure 6. Exogenous NO inhibited the afferent response to 5-HT and distention instead of CCK in LFF mice**

*A*, effects of NO donor SNP (10 $\mu$M) on jejunum afferent sensitivity to 5-HT (10 $\mu$M) in all units and subpopulations. SNP inhibited the afferent response to 5-HT in LFF mice ($P < 0.05$, two-way ANOVA with Bonferroni test, $N = 7$) instead of HFF mice. Inhibition was observed in LT afferents from both LFF ($P < 0.01$) and HFF ($P < 0.05$) mice. No significant change was seen in WDR afferents. *B*, CCK (100 nM) sensitivity was not affected by SNP in both LFF

and HFF mice, as well as in all subpopulations. *C* and *D*, the afferent response to distention was inhibited by SNP in LFF mice (*C*) ($P < 0.001$, two-way ANOVA with Bonferroni test, $N = 13$) but not in HFF mice (*D*) ($P = 0.522$, $N = 13$). *E*, the only subpopulation showing a significant change in the distention response was the WDR afferents of LFF mice ($P < 0.01$). *F*, the effects of SNP on 5-HT and CCK sensitivity of individual afferent units in LFF mice were diverse. LFF *vs.* LFF+SNP, HFF *vs.* HFF+SNP were repeated measurements in the same mice.

HFF mice in both the dark phase ($9.33 \pm 1.38$ *vs.* $7.89 \pm 1.83$ kcal, $P = 0.102$) and light phase ($2.24 \pm 1.29$ *vs.* $1.63 \pm 0.76$ kcal, $P = 0.231$), although this was not statistically significant.

A 3 week treatment of L-NIL was performed on HFF mice. Differences in weekly body weight gain and food intake between saline and L-NIL treated HFF mice were compared at three time points using an unpaired *t* test. Compared to saline-treated mice, total body weight gain was numerically lower in the L-NIL treated group ($2.6 \pm 1.4$ *vs.* $3.4 \pm 0.8$ g, $P = 0.171$, unpaired *t* test, $N = 8$) and weight gain after the first week was significantly reduced ($0.4 \pm 0.7$ *vs.* $1.1 \pm 0.5$ g, $P < 0.05$) (Fig. 7*D*). Energy intake (relative to the week prior to treatment) was significantly reduced in the first week ($88.2 \pm 6.7\%$ *vs.* $95.4 \pm 6.0\%$, $P < 0.05$, $N = 8$) (Fig. 7*E*). No significant difference was observed at weeks 2 and 3. In addition, epididymal fat pad weight, normalized to total body weight, was significantly lower in L-NIL treated mice at the end of 3 weeks ($4.60 \pm 1.24\%$ *vs.* $5.78 \pm 0.84\%$, $P < 0.05$, unpaired *t* test, $N = 8$) (Fig. 7*F*).

## Discussion

A poor understanding of the molecular mechanisms accounting for the development of obesity and hyperphagia impedes the progress of specific therapy. The present study has provided insight into a mechanism

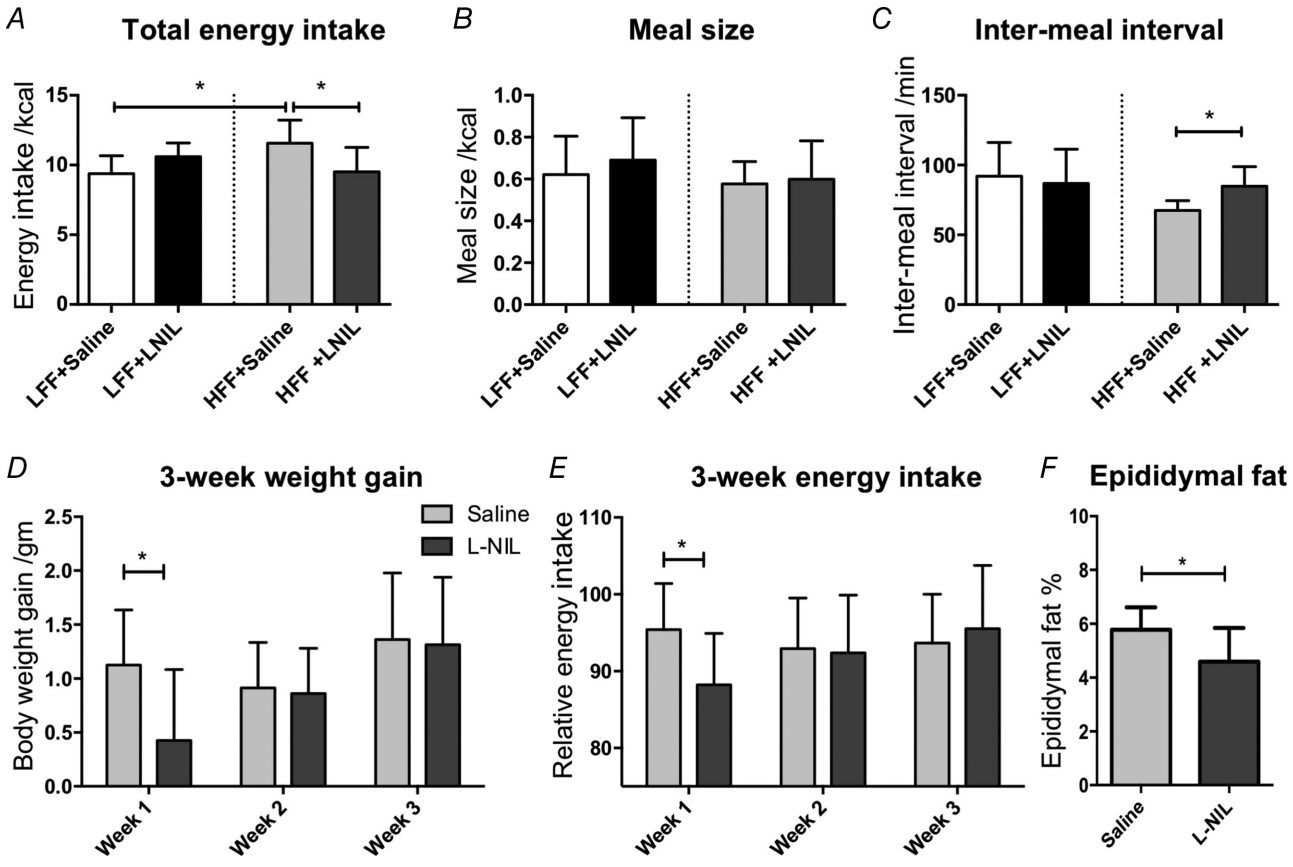


**Figure 7. iNOS inhibition reduced food intake and weight gain in HFF mice**
*A*, energy intake during 24 h after L-NIL injection (10 mg kg$^{-1}$) was significantly reduced compared to saline treatment in HFF mice ($P < 0.05$, two-way ANOVA with Bonferroni test, $N = 7$). *B*, no significant alteration in meal size was observed. *C*, inter-meal interval was increased by L-NIL in HFF mice ($P < 0.05$, two-way ANOVA with Bonferroni test). *D–F*, HFF mice were treated with L-NIL (10 mg kg$^{-1}$, daily I.P. injection) or saline for 3 weeks. L-NIL reduced body weight gain (*D*) ($P < 0.05$, unpaired *t* test, $N = 8$) and energy intake (*E*) ($P < 0.05$) in the first week of treatment. Percentage of epididymal fat was lower in L-NIL treated mice (*F*) ($P < 0.05$).

where elevated iNOS-derived NO impairs vagal afferent sensitivity via increasing $K_{2P}$ channel conductance, disrupts its control of food intake, and drives hyperphagia and obesity. By patch clamping, afferent recording and metabolic monitoring, we have provided evidence at the level of sensory neurons, afferent nerves and feeding behaviour, comprising inhibition of iNOS increased nodose neuron excitability and afferent sensitivity to satiety signals and distention, and reduced acute energy intake in obese mice. In addition, long-term suppression of iNOS in obese mice reduced visceral fat and decreased body weight gain, although the most prominent effect was in the first week.

iNOS is expressed in multiple cell types, such as macrophages (Lumeng *et al.* 2007), plasma cells (Fritz *et al.* 2012), epithelia cells (Kolios *et al.* 1998) and microglia cells (Sierra *et al.* 2014), and is probably signalling from multiple sources in the present study. A number of studies have revealed that a high-fat diet increases NO production or iNOS expression in many different sites, such as plasma (Stanimirovic *et al.* 2016), liver (Feng *et al.* 2016), kidney (Sherif, 2014), small intestine (Ou *et al.* 2012) and adipose tissue (Perreault & Marette, 2001), although not all studies have identified an increase (Perreault & Marette, 2001; Eccleston *et al.* 2011; Stanimirovic *et al.* 2016). The present study demonstrated that, after 6–8 weeks of high-fat diet feeding, the iNOS protein level was increased in nodose ganglia and jejunum from HFF mice, in association with increased NO production in plasma, small intestine and nodose neuron-incubated culture media. When combined with our electrophysiology results of both nodose ganglion neurons and peripheral intestinal afferents, this indicates that iNOS-derived NO originates from both peripheral and the nodose ganglion sites. Because iNOS is known to be stimulated by pro-inflammatory cytokines, our observation is consistent with the proposed low-grade chronic inflammation associated with obesity. Indeed, other studies have reported that elevated levels of lipopolysaccharides (LPS) are involved in obesity-induced changes in vagal afferent neurons (de La Serre *et al.* 2010; de Lartigue *et al.* 2011). Because LPS is a potent activator of iNOS, the results of the present study may provide a mechanistic explanation.

Our previous studies have demonstrated that obesity decreased the excitability of vagal afferent neurons (Daly and Park *et al.* 2011) as a result of increased $K_{2P}$ conductance (Park *et al.* 2018); however, the cellular mechanisms involved were unresolved. In the present study, measurement of electrophysiological membrane properties in nodose neurons revealed that administration of NOS and sGC inhibitors prevented the decreased excitability in HFF neurons. We also demonstrated that the inhibition of iNOS and sGC increased input resistance in HFF neurons, indicating a reduction in $K^+$ conductance. Correspondingly, the increased $K_{2P}$ conductance in HFF neurons was reduced by iNOS inhibitor. Thus, our data suggest that accumulation of iNOS-derived NO decreases excitability of vagal afferent neurons in obesity, at least in large part, by modulating $K_{2P}$ channels.

A previous study observed impaired afferent sensitivity to 5-HT, CCK and distention of the jejunum in HFF mice (Daly and Park *et al.* 2011). The present study proposed an iNOS–NO mediated mechanism to account for this impairment based on inhibition of iNOS potentiating afferent sensitivity to 5-HT, GLP-1, CCK and distention in the small intestine of HFF mice. This is consistent with a previous study in which iNOS inhibition increases the afferent response to 5-HT and distention on a mouse model of intestinal inflammation induced by indomethacin (Xue *et al.* 2009). Increasing satiety is one of the most important strategies for the pharmacological therapy of obesity. 5-HT agonists and GLP-1 agonists are two among five classes of drugs currently approved for obesity treatment in the USA (Shettar *et al.* 2017). The administration of a CCK-A agonist was not effective to induce weight loss in clinical trials to date (Kim *et al.* 2011), although enhanced endogenous CCK signalling might still be beneficial because it is well-established that CCK activates vagal afferents to inhibit food intake (Dockray, 2014).

Inhibition of iNOS in obesity recovered impaired satiety signalling and hence should reduce food intake. Indeed, we demonstrated that the hyperphagia present in HFF mice was reversed by iNOS suppression. We observed shorter inter-meal intervals but unaltered meal sizes in HFF mice compared to LFF mice. Changes in meal patterns caused by a high-fat diet have been variable in previous studies. One study suggested that a high-fat diet increased meal size and decreased inter-meal interval (Warwick *et al.* 2000), whereas another study reported increased meal frequency and reduced meal size on rats, with these changes adapting after long-term exposure to diet (Paulino *et al.* 2008). Many factors could influence meal patterns, such as the feeding pattern of mice (i.e. frequent feeding, particularly nocturnally), diet composition and energy density, and exposure time to the diet. Long-term suppression of iNOS decreased energy intake and body weight gain in the first week of treatment, and these effects diminished afterwards, implying takeover by compensative mechanisms. More importantly, visceral fat was reduced in treated mice at the end of treatment. One week of treatment in mice may be equivalent to a considerable period on humans; thus, the beneficial effects of iNOS suppression on obesity are still relevant. In addition, the observation at the end of the 3 week period of less visceral fat stores, yet a similar weight gain, suggests the possibility of increased lean body mass during treatment with L-NIL, although we did not measure this directly.

Previous studies addressing the involvement of iNOS in obesity are limited to insulin resistance. iNOS contributes

to obesity-related insulin resistance by reducing insulin receptor substrate-1 expression (Sugita *et al.* 2005). Consistently, iNOS knockout mice on a high-fat diet are protected from insulin resistance (Noronha *et al.* 2005; Zanotto *et al.* 2017). The present study clarified the involvement of iNOS in the pathogenesis of obesity in terms of modulation of satiety signalling conveyed by vagal afferents. Interestingly, iNOS knockout did not prevent the excessive weight gain induced by a high-fat diet (Noronha *et al.* 2005) because these models may be limited by compensatory mechanisms. However, these observations do not necessarily rule out the potential beneficial effects of iNOS inhibition on existing obesity.

NO has pleiotropic and complex roles in various physiological activities occurring in multiple systems, including the nervous system. Our exogenous NO data confirmed the inhibitory effects of NO on afferent sensitivity to 5-HT and distention, despite elusive effects on CCK sensitivity. Our single unit analysis revealed that NO had diverse effects on afferent sensitivity depending on the stimuli and target subpopulations. Other studies on non-obese models have also reported contrasting effects of NO (Kentish *et al.* 2014; Park *et al.* 2014). Further investigations are required to determine the complete pathway of the effects of NO on afferent nerve sensitivity.

In summary, obesity is a pandemic health issue with few tangible and safe treatment options. Many 'distal' causes of obesity, such as lifestyle, environment, occupation and social influences, are difficult to manipulate. Therefore, molecular pathways more proximal to the origins of obesity may be valuable therapeutic targets. The present study has provided insight into an iNOS–NO–vagal afferent pathway that accounts for the impairment of satiety signalling via vagal afferents in diet-induced obesity. Therefore, pharmacological manipulation of this pathway is a promising therapy for hyperphagia and obesity.

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## Additional information

### Competing interests

The authors declare that they have no competing interests.

### Author contributions

All experiments were performed in the Gastrointestinal Disease Research Unit at Queen's University in Kingston, ON, Canada. All authors approved the final version of the manuscript submitted for publication and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. MB supervised the project and obtained grant support. MB, SP and YY designed the research. YY performed the western blotting, afferent recording, feeding monitoring and relevant analyses. SP performed the patch clamp studies, as well as most of the nitrate assays and relevant analyses. YY, SP and MB interpreted the data, and drafted and revised the manuscript.

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## Translational perspective

Effective pharmacological treatments for obesity are limited as a result of a poor understanding of the molecular mechanisms underlying the development of obesity. Increasing satiety is one of the most important strategies, and 5-HT and GLP-1 agonists (both are satiety mediators) are two among five classes of drugs currently approved for obesity therapy in the USA. However, our previous investigations have demonstrated that obese mice have reduced vagal afferent sensitivity to satiety signals. Thus, the efficacy of exogenous satiety mediators may be limited. A low-grade inflammation occurs in obesity with increased expression of inducible nitric oxide synthase (iNOS). The present study hypothesized that elevated iNOS-derived NO impairs vagal afferent sensitivity and disrupts its control of food intake leading to hyperphagia and obesity. We found that inhibition of iNOS in obese mice restored vagal afferent neuronal excitability, increased the afferent response to satiety mediators and reduced short-term energy intake. A prolonged inhibition of iNOS reduced energy intake and body weight gain during the first week and also reduced amounts of visceral fat after 3 weeks. We identified a novel pathway underlying disrupted satiety regulation in obesity. This finding may drive more studies addressing its upstream and downstream pathways, such as mechanisms modulating iNOS overexpression, involvement of microbiota, possible targets of NO and corresponding subcellular signalling pathways, as well as beneficial effects on relevant metabolic functions. A better understanding of this pathway will promote clinical studies to develop specific treatments for hyperphagia and obesity.